SUBJECT: Review of the J12-03 data from the [redacted]" and the Proposed "Solazyme Inactivation Protocol to Simulate Actual Commercial Manufacture Operations"

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********** [redacted] **********

I. INTRODUCTION

Solazyme submitted inactivation data to the Agency (2-11-13) entitled [redacted]" and a proposed inactivation protocol entitled "Solazyme Inactivation Protocol to Simulate Actual Commercial Manufacture Operations" (2-11-13). There are various questions/concerns with the data in the drum drying study which, of course, carry over to the proposed inactivation protocol at commercial scale.

[redacted]

The process of demonstrating the effectiveness of an inactivation treatment for microorganisms is a difficult and complex task. Inactivation treatments such as steam, dry heat, or chemical disinfectants have different mechanisms of action. Each specific

procedure needs to be tested on the particular organism, and even the growth phase of the organism affects the kill rate. The number of microorganisms present also affects the microbial death kinetics. It takes longer periods of time to inactivate bacterial suspensions with greater densities of microorganisms that suspensions with fewer microorganisms (Tortora et al., 2008). Inactivation protocols need to be tested not only at laboratory scale, but also at scale-up under conditions that will actually be encountered with commercial production because of inconsistencies with mixing, clumping of cells, etc. In addition to the complexities associated with scale-up of inactivation treatments, robust efforts to resuscitate any remaining viable cells that may be merely damaged, but not dead, is necessary. It is well known that in nearly every population of microorganisms, there are some injured, but still viable, cells in addition to the viable or the completely inactivated (dead) cells (van Schothorst, 1975). These sublethally damaged cells are physiologically altered, and thus, need both more time and an appropriate nutritional environment to allow for repair before actual growth and multiplication can resume.

The absence of growth on an agar plate inoculated with a sample of inactivated microbial biomass is not sufficient to conclude 100% cell kill or whatever log reduction. Unless there have been rigorous attempts to provide optimal resuscitation conditions for any sublethally injured or stressed cells one cannot eliminate the possibility that the technique used to grow up any cells may actually be detrimental to sublethally damaged cells. Attempts to provide optimal resuscitation conditions have not been used in the inactivation studies/data provided thus far. [redacted]

There are also many specific modifications to the inactivation protocols steps that probably warrant discussion with the company prior to them conducting the proposed commercial-scale up studies. A discussion of inactivation techniques and resuscitation protocols follows.

II. PROPOSED INACTIVATION

[redacted]

[redacted]

III. DESICCATION AND DRY HEAT INACTIVATION

[redacted] There are numerous studies in the literature dealing with inactivation of bacteria, however, no such studies for algae could be found. Several studies have demonstrated that [redacted] are resistant to the heat treatments typically employed with pasteurization of milk. This is thought to be due to the resistant material in the cell wall known as sporopollenin. However, pasteurization involves heat transfer in an aqueous medium. It is known that there are differences in a microorganism's thermal resistance to moist heat and to dry heat (Ernst, 1968).

The efficacy of dry heat inactivation is unknown for this alga. Also, the existence of a dormant spore stage in [redacted] must also be considered as spores typically are more difficult to inactivate compared to vegetative cells. During reproduction of [redacted]

Much of the information on inactivation/sterilization techniques is data from studies in the early and mid 1900's. The comprehensive chapter of Rahn (1945) covers many of these studies, and it is often referenced, even in more current articles.

Desiccation of bacteria does not always result in cell death. According to Rahn (1945), there are two considerations: 1) the number of fatalities due to the removal of moisture, and 2) the gradual death of those bacteria that survive from the moist to the dry state. He says that even modern drying methods such as spray-drying leaves only a very thin protective layer around the bacterial cells, however, many still survive. Apparently, the most important aspect of desiccation is the medium in which the bacteria are present during the drying treatment. If the bacteria are dried in their culture media, such as broth or milk, they survive fairly well, whereas there are few survivors with washed bacterial cells. Otten (1930; as cited by Rahn, 1945) found that the depth of the layer of dried cells was quite important to survival. Of course, the thinner the layer, the smaller number of survivors. Otten said that the bacterial proteins protect the cells by acting as a protective colloid which lessens the abruptness of the drying process, and not by forming a protective cover over the cell that prevents complete desiccation.

[redacted] According to Rahn (1945) in a discussion of powdered milk "even the severe treatment of the drum-drying method, where the milk flows onto rotating, steam-heated drums and is scraped off a paper-thin, dry sheet, leaves some vegetative forms alive". He cites a paper by Supplee and Ashbaugh (1922) that said usually only 1 in 10,000 bacteria survive. However, others have found that with a rapid spray-drying process, there are a greater number of survivors. According to one reference cited by Rahn (Hunziker, 1935) the bacterial plate counts of drum-dried milk powder ranged from 45 - 500,000 per gram, whereas with the spray-dried milk powders, there were greater numbers of survivors with plate counts of 4,400 - 5,500,000 per gram milk powder. Rahn also cites the work of Crossley and Johnson (1942) who found ranges of 200 to 19,500,000 bacteria per gram in milk powders. It is unknown if these bacteria were bacilli endospores, vegetative cells, or both. Also, even though bacteria, sometime pathogens, can be found in powdered milk or infant formula, perhaps, [redacted]

With drum dryers, the bacteria die when in direct contact with the metal surface. The moisture in whatever product is being dried heats up and is driven off through evaporation. However, direct contact with the hot metal is apparently necessary for the microorganisms to be killed. Therefore, the thickness of the film on the drums affects whether all the microorganisms actually contact the metal. Rahn (1945) says that even in the manufacture of coins, there are still many bacteria on the coins because there is a protective layer of dirt preventing the direct contact between bacteria and the hot metal, and thus, the cells are not killed.

[redacted]

Death by dry heat is from oxidation when the temperature is raised above the maximal temperature of the species (Rahn, 1945). Rahn states that the dry cells do not display life functions, the enzymes are not active in the absence of moisture, and endogenous catabolism ceases. However, the dry enzymes retain their activity because there is no

coagulation of proteins. Dry proteins do not coagulate when heated to 100 ºC (Rahn, 1945).

Dry heat sterilization has several disadvantages. As presented by Ernst (1968), these are:

1. Heating is slow - diffusion and penetration of heat is slow due to the fact that the heat transfer medium (air) is very poor;
2. It requires long sterilizing periods - long exposure times are required because the killing rate by dry heat is slow, and heat absorption is slow as well;
3. It requires high temperatures;
4. Materials are damaged - deterioration of material occurs due to oxidation as dry heat killing is an oxidation process; and
5. There is a tendency to stratify - the severe tendency to stratify over a considerable range of temperatures must be overcome.

Ernst (1968) also states the heat availability affects the efficacy of the heat treatment as it relates to heat conductivity. Air is often chosen as the heat treatment rather than steam since it is the least expensive, but "air is not the best heat transfer medium". According to Ernst, "heat transfer can be accelerated by moving the air streams, since a grave limitation in dry heat sterilization by static means is severe stratification and lack of diffusion in and around relatively cool materials".

The presence of the medium in which the microbial cells exist is also important with the efficacy of inactivation treatments. The presence of organic materials in a matrix of cells has actually been shown to have a protective effect on dry heat inactivation of microorganisms. Rasmussen et al. (1964) assessed the dry heat resistance of *Salmonella* in rendered animal by-products. When free *Salmonella* cells were added to the meal just before heating, a temperature of 155 ºF (68.3 ºC) was sufficient to ensure sufficient destruction of the microorganisms. However, with a meal naturally contaminated with *Salmonella,* a higher temperature of 170 ºF (76.7 ºC) was needed to kill the bacteria. In another contaminated meal with an unusually high fat content of 13%, an even higher temperature treatment of 190 ºF (87 ºC) for 14 minutes still did not kill all the bacteria. Apparently, the meal, especially the one with the high fat content, afforded some protection from the dry heat to the bacteria. It is unknown how the high concentration of [redacted] production organism will affect its temperature sensitivity. [redacted]

Studies conducted by Mackey and Derrick (1982) showed that the lag time for repair of sublethally damaged cells of *S. typhimurium* differed depending on the stress, be it heating, freezing, drying, and gamma-radiation. Dried cells showed only a slight increase in lag time as viable numbers declined. The heated, frozen, and irradiated cells showed increasing lag times with decreasing survival, but the rate on increase was much greater with heated and frozen cells.

Rahn (1946) reported data of Otten (1930) that showed that dried bacteria were able to endure very high temperatures compared to vegetative cells. Dried typhoid (*Salmonella typhi*), dysentery (bacilli, Shigellosis, amoebic), and cholera (*Vibrio cholera*) bacteria, none of which are sporeformers, could survive at 58-60 ºC for 7 to 10 days, and even survived at 100 ºC for 1 to 2 hours. Several studies have found that higher temperatures were needed for cell kill with dry air versus that with moist heat. Ayers and Mudge (1921) investigated the inactivation of *E. coli* and a heat-resistant lactic type by drying aqueous suspensions of the bacteria. The temperature required for a 30-minute kill time was 60 ºC in milk, but it was 71-82 ºC in hot air. The heat resistant strains needed 76 ºC in milk, but 110 ºC in hot air for the 30-minute kill. According to Tortora et al. (2008), a temperature of 170 ºC for 2 hours is needed for sterilization of objects by dry heat.

It is well known that the inactivation of spores is much more difficult compared to vegetative cells of microorganisms. However, there may be differences in thermal resistance to moist heat than to dry heat, and a spore resistant to one type may not be resistant to the other and vice versa(Ernst, 1968). Also spores from different species are affected differently. It took <1 minute at 121 ºC to inactivate 1 million spores of *B. subtilis,* but it required 12 minutes of exposure to the same temperature to inactivate 100,000 spores of *B. stearothermophilus* (Ernst, 1968). Data on heat inactivation of spores of *Clostridium botulinum* showed necessary inactivation times at 120 ºC of up to >120 minutes (Tanner and Dack, 1922; as presented in Rahn, 1945). It was proposed by Black and Gerhardt (1962; as cited by Pflug and Schmidt, 1968) said that “the core of the dormant spore exists as an insoluble and heat-stable gel, in which cross-linking between macro molecules occurs through stable but reversible bonds so as to form a high-polymer matrix with entrapped free water”.

The inactivation of spores also depends on the matrix in which they exist. According to Pflug and Schmidt (1968), when spores are added to or trapped in solids, the suspending material affords protection to the spores resulting in increased heat resistance. [redacted]

IV. RESUSCITATION OF SUBLETHALLY INJURED CELLS

It has long been known that inimical treatments, both physical and chemical such as heat, freezing, disinfectants, etc., can leave a small portion of bacterial cells sublethally injured rather than killed. According to van Schothorst (1975) these partly inactivated cells “show an extended lag phase, are more exacting in their nutritional requirements or are sensitive to substances which uninjured cells of the same population fully tolerate” such as bile salts or sodium chloride. This knowledge of sublethally injured cells has prompted concern for the presence of damaged, but still viable, food-borne bacterial pathogens, especially on meats. To ensure food safety, the resuscitation of sublethally injured bacterial pathogens on foods has long been studied.

There are many intricacies involved with resuscitating injured cells. According to Martin et al. (1976), it is well established that the physiology of injured but viable cells differs from that of undamaged cells. Hence, injured cells are commonly unable to grow on standard agar media typically used for their isolation. In more recent years, it has also been widely acknowledged that dormant bacterial cells, in a stress-induced state known as the viable but nonculturable (VBNC) state, also cannot be resuscitated on rich agar media usually used for their cultivation in the laboratory (Oliver, 1999). The number of bacteria known to exist in the VBNC state is constantly growing, but was around 60 bacteria from 30 or so different genera including many human pathogens as of 2005 (Oliver, 2005). Determination of the reasons for the inability of these dormant cells to grow on agar media from this state has prompted much research into resuscitation strategies which may be relevant to the current discussion of resuscitation of injured cells. The use of a rich media for resuscitation is thought to be inappropriate, and there is an additional problem with the use of solid agar plates even of the same growth medium.

It has been known for more than half a century that resuscitation of sublethally injured cells was not achieved easily on agar plates unless attempts were made to eliminate hydrogen peroxide in the agar medium. As far back as 1956, Barry et al. said that it had been observed that small inocula of *Mycobacterium tuberculosis* grew poorly in synthetic media unless one of a variety of hydrogen peroxide-destroying agents, such as hemoglobin, serum fractions, charcoal, or cobalt was added to the medium. They examined Proskauer and Beck-type culture media and found that, as prepared, they contained between 1 - 10 µg/ml of H_2O_2. Apparently, media containing both citrate and manganese produce substantial quantities of hydrogen peroxide when autoclaved. In the absence of manganese, no peroxide was formed. However, only trace amounts of manganese were needed to result in peroxide production, and it was thought that such trace amounts were usually present in the asparagine used in the media.

Martin et al. (1976) also found that the use of sodium pyruvate and the addition of catalase to trypticase soy broth (TSB) enabled recovery of heat-injured *Staphylococcus aureus* by preventing the accumulation of hydrogen peroxide in and around the cells. Addition of heat-inactivated catalase did not enable recovery of the injured cells. They also found increased recovery of injured *Pseudomonas fluorescens, Salmonella typhimurium,* and *Escherichia coli.* Sublethal heat treatment to *S. aureus* has been shown to produce a variety of repairable damages (Iandolo & Ordal, 1966). These include strand breaks in the deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) (Gomez & Sinskey, 1973; Tomlins & Ordal, 1971; as cited by Martin et al., 1976). An additional damage associated with heat inactivation of microorganisms is the loss of function of the catalase enzyme.

Raymond et al. (1978) reported that more than 90% of heat-stressed *Salmonella senftenberg* cells failed to grow on trypticase soy agar (TSA) unless it was supplemented with compounds capable of degrading hydrogen peroxide. A 1% sodium pyruvate solution in the TSA produced maximal growth of heat-damaged cells. Also, the addition of other peroxide-degrading agents such as $FeSO_4^-$ and $KMnO_4^-$ (filter

sterilized – not autoclaved) also resulted in greater than 100 times higher counts of the heat-injured cells than on TSA alone. Likewise, McDonald et al. (1983) found that compounds that degrade or prevent the formation of hydrogen peroxide were necessary for recovery of both heat- and freezing-injured *E. coli* cells in tryptone-glucose extract agar. Likewise, superoxide dismutase or catalase in the media offered protection of thermally stressed and nonstressed cells, but moreso for the thermally stressed cells (Bucker and Martin, 1982). Mizuone et al. (2000) found that the addition of catalase or sodium pyruvate allowed for resuscitation of viable but nonculturable cells of *Vibrio parahaemolyticus.*

It has also been shown that cells in the stationary phase are much more thermotolerant than exponentially growing cells. Hengge-Aronis et al. (1991) found that in *E. coli,* the transcription of the regulatory gene, *rpoS,* which is induced during entry into the stationary phase in rich media controls glycogen synthesis, thermotolerance, hydrogen peroxide resistance, long-term starvation survival, the induction of a number of different proteins, and also affects cell size and cell shape in stationary phase growth. Therefore, it is important that the cells be inactivated while in exponential growth phase rather than stationary phase.

[redacted] As has been shown by Duffy et al. (1995) and Aldsworth et al. (1998), the presence of a competitive microflora at high densities (10^8 colony-forming units (cfu)) resulted in greatly enhanced survival of the populations of *S. typhimurium* against mild heat (55ºC) or freeze injury. Dodd et al. (1997) also found that the addition of exponential growth phase competitor cells at a level of 10^8 cfu resulted in an increased level of resistance of a 10^5 cfu population of *S. typhimurium* similar to the thermotolerance observed with stationary growth phase cultures. They found that the particular genera of the added bacteria didn't matter as long as the cells were alive. Thus, the enhanced resistance effect was shown not be a simple one such as the presence of a physical protective protein layer that could be provided by dead cells as well. In a latter publication by some of these authors (Bloomfield et al., 1998) it was speculated that during resuscitation, exposure of growth-arrested cells (such as VBNC) to high nutrient levels would result in an imbalance of metabolism which could result in the production of super-oxide and other free radicals almost spontaneously (Oliver, 1999). Without allowing the cells to perform some pre-adaptation strategies such as producing starvation-induced stress proteins, the cells would be incapable of detoxifying the free radicals and be killed (Oliver, 1999). Bloomfield et al. (1998) claimed that it is the devastatingly destructive power of oxidative damage from the metabolic imbalance resulting from rich media that kills the VBNC cells. It seems likely that the same oxidative destruction seen with VBNC cells could also occur with sublethally damaged cells that also require repair before resuming normal metabolic activity.

In a review of recent findings about the VBNC state in bacteria, Oliver (2010) discusses some theories on resuscitation of VBNC cells. For some bacteria such as vibrios, a simple reversal (temperature increase) of the stress (temperature downshift)

that caused the cells to enter the VBNC state allowed for resuscitation of the cells. It has also been found that extracellular bacterial proteins known as 'resuscitation-promoting factors' (Rpfs) occur in various organisms such as *Mycobacterium tuberculosis* and *M. smegmatis* which were found to be petidoglycan hydrolases involved with cell wall digestion and cell division. Other resuscitation factors have been reported for a number of Gram-positive and Gram-negative species when incubated in media containing human norepinephrine. A quorum-sensing system for resuscitation has also been suggested (Oliver, 2010). There is no literature suggesting that such VBNC state occurs in green algae. However, it is known to occur in cyanobacteria (has been suggested for any green algae

A report was published by the New Zealand Food Safety Authority in 2008 entitled "Resuscitation of putative viable but non-culturable (VNC) food-borne bacteria of significance to New Zealand". It lists a number of different resuscitation strategies tried by various researchers. In addition to the use of a peroxide-degrading compound in agar media, it has also been proposed that one attempt to resuscitate VNC cells in minimal media rather than in nutrient-rich media (Wong et al., 2004). They found that there was a 12-hr lag period with resuscitation of VBNC cells on TSB that did not occur with the minimal media. Whitesides and Oliver (1997) also reported that resuscitation appears to occur only when little or no nutrient is present, even though the rich media was not actually toxic to the *V. vulnificus*. Perhaps, this strategy of attempting resuscitation on minimal or diluted media addresses the oxidative destruction found to occur with the use of rich media.

Trypticase soy broth, and thus the solid version as trypticase soy agar, is a medium rich in carbohydrates, amino acids, vitamins, and minerals. These components are supplied in the form of casein peptone (from pancreatic digestion) and soya peptone (from papanic digestion) along with sodium chloride for osmotic balance.

Although much of the research done on resuscitation of microorganisms has been done for bacteria, it seems likely that similar phenomena may pertain to any heterotrophic microorganism, even an alga. The formation of hydrogen peroxide during the heating of the agar media may also be problematic for enumeration of damaged/stressed algal cells. Likewise, if there is injury resulting from a metabolic imbalance leading to oxidative damage and cell death when taking bacterial cells from an injured/stressed state into rich media, it seems plausible the same thing may happen with a heterotrophic alga.

A robust effort for resuscitation of sublethally damaged cells under optimal conditions for recovery should be attempted for the inactivation treatments proposed by Solazyme to provide a level of certainty that the proposed inactivation treatments are successful in killing all viable cells, and not merely sublethally damaging them.

V. SPECIFIC PROBLEMS WITH THE INACTIVATION DATA AND PROPOSED INACTIVATION PROTOCOL

A. Inactivation Data Supplied

1. [redacted]

2. [redacted]

3. Need robust resuscitation efforts - with the use of a peroxide-degrading agent (e.g., pyruvate, catalase, superoxide dismutase) and into dilutions of TSA/glucose media (perhaps full strength, and dilutions of 1:10, 1:100, even perhaps 1:1000).

B. Proposed Large-Scale Inactivation Protocol

1. [redacted]

2. I do not see the utility of sampling three time points with each inactivation step - [redacted]

3. As above, the plating assay should address peroxide formation. For the pre-enrichment, there should be inoculations into diluted media as well as full-strength.

4. If growth occurs, the O.D. may need to be read more frequently than daily. Do not do O.D. measurements on dilutions of a sample - just read it more often.

5. With first experiment - plating only - see above - also is 1 g into 50 ml too dilute? Don't expect to have many cells - so perhaps 1 g into 9 ml (like done with soils to approximate a 1:10 dilution) may be more appropriate.

6. With the second experiment, pre-enrichment inoculations should be into diluted media as well as full strength.

7. Again, if plating the enrichment cultures, address peroxide issue going from diluted media to full strength plates (probably OK if full strength to full strength).

8. Since samples will be contaminated bacteria since the processing/inactivation steps are not sterile, would the use of [redacted] be useful?

9. I recommend that the resuscitation attempts be determined on a lab-scale basis first. Once there is convincing evidence of cell death by the inactivation treatments at the bench scale, then the determination of their effectiveness at pilot and commercial-scales should be addressed.

REFERENCES

Aldsworth, T.G., R.L. Sharman, C.E.R. Dodd, and G.S.A.B. Stewart. 1998. A competitive microflora increases the resistance of *Salmonella typhimurium* to inimical processes: Evidence for a suicide response. Appl. Environ. Microbiol. 64:1323-27.

[redacted]

Barry, V.C., M.L. Conalty, J.M. Denneny, and F. Winder. 1956. Peroxide formation in bacteriological media. Nature 178:596-7.

Bloomfield, S.E., G.S.A.B. Stewart, C.E.R. Dodd, I.R. Booth, and E.G.M. Power. 1988. The viable but nonculturable phenomenon explained? Microbiol. 144:1-3.

Bucker, E.R. and S. E. Martin. 1982. Effect of free-radical scavengers on enumeration of thermally stressed cells of *Staphylococcus aureus* MF-31. Appl. Environ. Microbiol. 43:1020-25.

[redacted]

Dodd, C.E.R., Shaman, R.L., Bloomfield, S.F., Booth,I.R. & Stewart, G.S.A.B. 1997. Inimical processes: bacterial self-destruction and sub-lethal injury. Trends Food Sci Technol. 8:238-41.

Duffy, G., A. Ellison, W. Anderson, M.B. Cole, and G.S.A.B. Stewart. 1995. Use of bioluminescence to model the thermal inactivation of *Salmonella typhimurium* in the presence of a competitive microflora. Appl. Environ. Microbiol. 61:3463-65.

[redacted]

Hengge-Aronis, R., W. Klein, R. Lange, M. Rimmele, and W. Boos. 1991. Trehalose synthesis genes are controlled by the putative sigma factor encoded by *rpoS* and are involved in station-phase thermotolerance in *Escherichia coli.* J. Bacteriol. 173:7918-24.

[redacted]

[redacted]

[redacted]

Martin, S.E., R.S. Flowers, and Z.J. Ordal. 1976. Catalase: Its effect on microbial enumeration. Appl. Environ. Microbiol. 32:731-34.

McDonald, L.C., C.R. Hackney, and B. Ray. 1983. Enhanced recovery of injured *Escherichia coli* by compounds that degrade hydrogen peroxide or block its formation. Appl. Environ. Microbiol. 45:360-65.

Mizuone, Y., S. N. Wai, T. Ishikawa, A. Takade, and S. Yoshida. 2000. Resuscitation of viable but nonculturable cells of *Vibrio parahaemolyticus* induced at low temperature under starvation. FEMS Microbiol. Letters 186:115-20.

New Zealand Food Safety Authority. 2008. Resuscitation of putative viable but non-culturable (VNC) food-borne bacteria of significance to New Zealand. Draft Final Report. Institute of Environmental Science & Research Limited, Christchurch Science Center, Christchurch, New Zealand (www.esr.cri.nz).

Oliver, J.D. 1999. The viable but nonculturable state and cellular resuscitation. *In*: C.R. Bell et al. (eds). Proceeding of the 8th International Symposium on Microbial Ecology, Atlantic Canada Society for Microbial Ecology, Halifax, Canada.

Oliver, J.D. 2010. Recent findings on the viable but nonculturable state in pathogenic bacteria. FEMS Microbiol. Rev. 34:415-25.

[redacted]

Rayman, M.K., B. Aris, H.B.El Derea. 1978. The effect of compounds which degrade hydrogen peroxide on the enumeration of heat-stressed cells of *Salmonella senftenberg*. Can. J. Microbiol. 24(7):883-85.

van Schothorst, M. 1975. Resuscitation of sublethally impaired cells. Antonie van Leeuwenhoek 41(1):380-81.

Whitesides, M.D. and J.D. Oliver. 1997. Resuscitation of *Vibrio vulnificus* from the viable but nonculturable state. Appl. Environ. Microbiol. 63:1002-5.

Wong, H.C., P. Wang, S.Y. Chen, and S.W. Chiu. 2004. Resuscitation of viable but nonculturable *Vibrio parahaemolyticus* in a minimal salt medium. FEMS Microbiology Letters 233:269-75